Cell, Vol. 87, 85–94, October 4, 1996, Copyright ©1996 by Cell Press

The Major Cytoplasmic Histone Acetyltransferase in Yeast: Links to Chromatin Replication and Histone Metabolism

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Summary

We have isolated the predominant cytoplasmic histone acetyltransferase activity from Saccharomyces cerevisiae. This enzyme acetylates the lysine at residue 12 of free histone H4 but does not modify histone H4 when packaged in chromatin. The activity contains two proteins, Hat1p and Hat2p. Hat1p is the catalytic subunit of the histone acetyltransferase and has an intrinsic substrate specificity that modifies lysine in the recognition sequence GXGKXG. The specificity of the enzyme in the yeast cytoplasm is restricted relative to recombinant Hat1p suggesting that it is negatively regulated in vivo. Hat2p, which is required for high affinity binding of the acetyltransferase to histone H4, is highly related to Rbap48, which is a subunit of the chromatin assembly factor, CAF-1, and copurifies with the human histone deacetylase HD1. We propose that the Hat2p/Rbap48 family serve as escorts of histone metabolism enzymes to facilitate their interaction with histone H4.

Introduction

The DNA of eukaryotic chromosomes is packaged with proteins as chromatin, with the fundamental repeating subunit being the nucleosome. Approximately 145 base pairs of DNA are wrapped 1.75 times around the core histone octamer, which is comprised of two molecules each of histones H2A, H2B, H3, and H4 (van Holde, 1989). The basic nucleosome core is conserved throughout eukaryotes and plays a critical role in chromosomal processes, such as gene regulation, chromosome condensation, recombination, and replication.

During replication, the DNA strands as well as the chromatin components are duplicated. After passage of a replication fork, parental histones reassociate with either of the two sister DNA helices (Sogo et al., 1986). But in addition, new histones must be synthesized and deposited onto the newly replicated DNA in order to restore the full complement of chromatin on both sister chromatids. DNA replication and the assembly of chromatin are necessarily highly coordinated. However, the mechanistic pathway that leads from the synthesis of histones in the cytoplasm to their assembly into chromatin at sites of DNA replication is poorly understood (Krude, 1995).

High levels of histone synthesis occur as DNA replication begins (Wu and Bonner, 1981). Shortly after their synthesis, the histones are modified in the cytoplasm. The most notable modification is the acetylation of the ϵ-amino groups of lysine residues located near the NH_2 termini of the histones (Ruiz-Carrillo et al., 1975). These modified histones enter the nucleus and are deposited onto the newly replicated DNA in what appears to be a two-step process. Histones H3 and H4, as a tetramer, are deposited first, followed by addition of two H2A/H2B heterodimers to complete the nucleosome (Worcel et al., 1978; Stillman, 1986; Perry et al., 1993). Studies of histone H4, the most evolutionarily conserved of the histones, provide compelling evidence that cytoplasmic acetylation plays a critical role in the assembly of newly synthesized histones into chromatin. Acetylation of histone H4 in the cytoplasm is nonrandom and evolutionarily conserved. In the three diverse eukaryotes where it has been examined in vivo, only two of four lysine residues that can potentially be acetylated are actually modified on newly synthesized histone H4. The lysines at positions 5 and 12 are acetylated in Drosophila and humans, and in Tetrahymena, the homologous residues 4 and 11 are used (Chicoine et al., 1986; Sobel et al., 1995).

A mechanistic link between cytoplasmic acetylation of histones and chromatin deposition is provided by CAF-1, a three-subunit protein complex (p150, p60, and p48) originally identified in human cell nuclear extracts that mediates the in vitro assembly of histone H3/H4 tetramers specifically onto actively replicating DNA (Stillman, 1986; Smith and Stillman, 1989; Kaufman et al., 1995). CAF-1 selectively utilizes cytoplasmic, acetylated histone H3/H4 as a substrate for chromatin assembly but does not assemble histones isolated from nuclei.

An understanding of the enzymes responsible for histone acetylation is critical for elucidating the details of chromatin assembly. Enzymatic activities that catalyze the transfer of an acetyl group from acetyl coenzyme A to the lysine residues in the NH_2-terminal tails of the core histones have been characterized as histone acetyltransferases (HATs; reviewed in Brownell and Allis, 1996). Based on preliminary biochemical characterizations, HATs are divided into two classes. The HAT A enzymes are located in the nucleus and are capable of acetylating the core histones when present in nucleosomes (Brownell and Allis, 1996). These enzymes are thought to be responsible for generating the histone acetylation patterns that correlate with transcriptionally active chromatin. The recent discovery that yeast Gcn5p, which had previously been isolated genetically as a transcriptional regulatory protein, is a type A HAT demonstrates a direct link between nuclear histone acetylation and transcriptional regulation (Brownell et al., 1996).

HAT B enzymes are cytoplasmic and specific for free histones. These enzymes are thus excellent candidates for the cytoplasmic acetylation implicated in chromatin assembly during DNA replication (Brownell and Allis, 1996). In order to better understand the role of histone

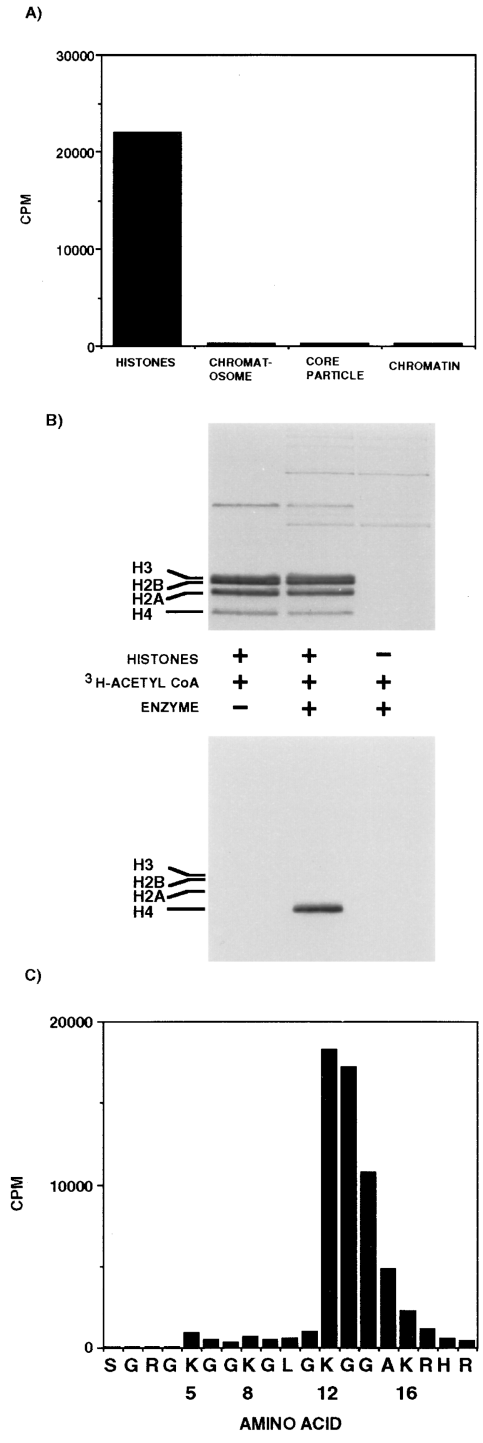

Figure 1. A Cytoplasmic Histone Acetyltransferase Activity Specific for Lys-12 of Histone H4

(A) After partial purification, cytoplasmic HAT activity was incubated with ^{3}H-acetyl CoA and equal concentrations of chicken erythrocyte histones (HISTONES) or histones associated with DNA as nucleosomal arrays (CHROMATIN), a CHROMATOSOME, or a nucleosomal CORE PARTICLE. The bar graph represents the amount of ^{3}H-acetyl groups (CPM) transferred to histone in each form.

(B) The same cytoplasmic HAT activity was incubated with chicken erythrocyte histones as in (A) and the reaction products were resolved by SDS–PAGE. The gel was stained with Coomassie blue (top panel) and then analyzed by fluorography to visualize ^{3}H-labeled peptides (bottom panel). The mobility of each of the core histones is indicated along the left-hand side of the panels.

(C) The cytoplasmic HAT activity was incubated with ^{3}H-acetyl CoA and a synthetic peptide that contains the NH_2-terminal 28 amino acids of yeast histone H4. The peptide was purified and subjected to NH_2-terminal peptide sequencing (X axis; single letter amino acid code). The amount of ^{3}H incorporated in each residue was determined (CPM).

acetylation in chromatin assembly, we have purified and characterized a type B HAT from S. cerevisiae. Our findings provide new insights into histone acetylation and a link between acetylation and chromatin assembly.

Results

A Histone Acetyltransferase B Activity in Yeast Cytoplasmic Extracts

Nuclear and cytoplasmic extracts from S. cerevisiae cells were made and subjected to a HAT assay. Extracts were incubated with ^{3}H-acetyl CoA and highly purified chicken erythrocyte histones, and transfer of the ^{3}H-acetyl moiety to histones by a HAT activity was monitored. Chicken erythrocyte histones are an excellent substrate in this assay because they are readily purified to homogeneity (Feng et al., 1993) and largely underacetylated (Brotherton et al., 1981), and the two inner core histones, H3 and H4, are virtually identical to the yeast H3 and H4 histones (van Holde, 1989).

By the criteria of this assay, there are several HAT activities in both nuclear and cytoplasmic extracts (data not shown; Lopez-Rodas et al., 1989). A major activity found in the cytoplasm that was stable during purification procedures was analyzed further. Following fractionation of the extract by DE-52 anion exchange chromatography, the activity was characterized as to whether it was an A- or B-type HAT. Figure 1A shows that the cytoplasmic HAT readily acetylates histones free in solution, but has no activity when histones are complexed with DNA, as core particles, chromatosomes, or chromatin (van Holde, 1989).

To determine which of the free histones was acetylated by the cytoplasmic HAT activity, products of these reactions were analyzed on SDS-polyacrylamide gels. Only histone H4 was labeled by the ^{3}H-acetyl group (Figure 1B). Thus, the yeast cytoplasmic HAT activity is highly specific for free histone H4.

The residues of histone H4 that were acetylated by the cytoplasmic HAT activity were determined by using a synthetic peptide encoding the NH_2-terminal 28 amino acids of yeast histone H4 as a substrate. The acetylated peptide was then subjected to NH_2-terminal protein sequencing and the amount of radioactivity in each residue was determined. As shown in Figure 1C, the lysine at position 12 was the only site modified.

The substrate specificity exhibited by this cytoplasmic acetyltransferase activity was consistent with it being a type B HAT. It acetylated free histone H4 at lysine-12, which is one of the two lysine residues (5 and 12) where cytoplasmic acetylation has been detected in other eukaryotes (Chicoine et al., 1986; Sobel et al., 1995).

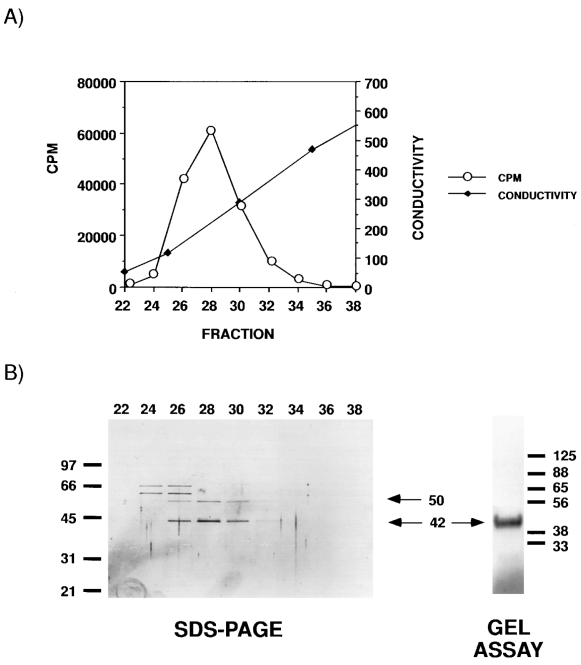

Figure 2. The Cytoplasmic Histone Acetyltransferase Activity Binds to the N-Terminal Tail of Yeast Histone H4

(A) The elution profile of the cytoplasmic HAT activity from a histone H4 tail affinity column. Aliquots (1.5%) of column fractions were incubated with ^{3}H acetyl CoA and purified histones. The amount of ^{3}H incorporated into histones is indicated by open circles (CPM). Conductivity of fractions is given as μS/cm^2 of a 1/200 dilution of fractions into water.

(B) Aliquots (7.5%) of even numbered fractions in (A) were separated by SDS 10% polyacrylamide gel electrophoresis and visualized by silver staining (left panel). The right panel is the autoradiogram from a lane of a HAT gel assay (GEL ASSAY) that contained a column fraction with high levels of HAT activity. Arrows between the panels show the apparent molecular weight of the two polypeptides that copurify with the HAT activity. The mobility of molecular weight standards are indicated in kDa.

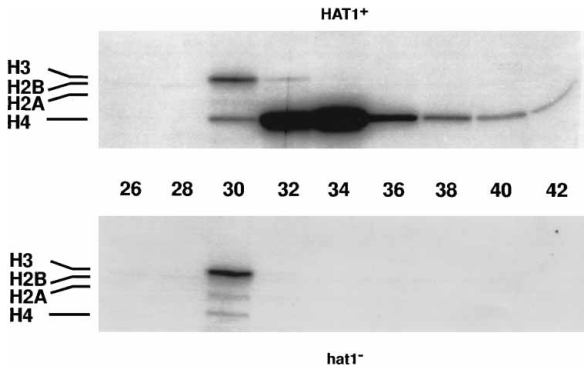

Figure 3. HAT1 Is the Major Cytoplasmic Histone Acetyltransferase Activity

Cytoplasmic extracts, made from a wild-type yeast strain (HAT1$^+$) and a strain with the *HAT1* gene deleted (hat1$^-$), were chromatographed on DE-52 anion exchange columns. Fractions from the columns were incubated with ^{3}H-acetyl CoA and chicken erythrocyte histones, and the reaction products were resolved by SDS–PAGE and fluorography. Fractions from the two columns had equivalent conductivities.

Purification of a Type B Histone Acetyltransferase

The initial steps in the purification of the yeast HAT B activity employed a variety of conventional chromatographic techniques based on anion exchange, size exclusion, chromatofocusing, and hydrophobic interactions (Experimental Procedures). The final purification steps involved affinity chromatography using a column with a synthetic peptide encoding the NH_2-terminal 28 amino acids of yeast histone H4 covalently coupled to Sepharose beads. The HAT B activity bound tightly to this resin and was eluted with high salt (Figure 2A).

Two polypeptides, with molecular weights of 50 kDa and 42 kDa, precisely coeluted with the HAT B activity (Figure 2B). To determine whether either of the two polypeptides alone was capable of catalysis, a HAT activity gel assay was carried out (Brownell and Allis, 1995). As can be seen in Figure 2B, the 42 kDa polypeptide contained the catalytic site of the HAT activity.

Two proteolytic fragments of the 42 kDa protein were isolated and sequenced. Matches to both were found in a single open reading frame (ORF) located on chromosome 16 of S. cerevisiae. To verify that the ORF encodes a HAT, it was cloned and expressed in E. coli and shown to confer HAT activity to E. coli extracts (data not shown and Figure 8). This ORF was identical to *HAT1* (*h*istone *a*cetyl*t*ransferase 1), a gene recently identified as having HAT activity (Kleff et al., 1995).

HAT1 Is the Major Cytoplasmic Histone Acetyltransferase Activity in Yeast

A null mutant of *HAT1*, created by deleting its protein coding region, had no apparent phenotypic consequence in a haploid strain (data not shown; Kleff et al., 1995). Cytoplasmic extracts made from *hat1* (null mutant) and *HAT1* (wild-type) strains were compared for HAT activity. The two extracts were each fractionated on a DE-52 column: for the wild-type extract there was a high level of histone H4 acetylating activity centered at fraction 34 (Figure 3, top panel). There was also a histone H3 acetylating activity primarily in fraction 30 (Figure 3, top panel). From the *hat1* extract, the strong histone H4 acetylating activity was completely absent (Figure 3, bottom panel). However, the histone H3 acetylating activity was not affected and a histone H4 acetylating activity coelutes with it in the absence of *HAT1* (Figure 3, bottom panel). A third polypeptide was also acetylated. Its migration is consistent with it being an NH_2-terminal proteolytic fragment of histone H3 (data not shown; Figure 3). The results of these experiments indicate that *HAT1* is the predominant cytoplasmic HAT in yeast.

Identification of the Second Acetyltransferase Subunit, HAT2

In addition to Hat1p, a polypeptide of ~50 kDa copurified with the HAT activity (Figure 2B). As done for Hat1p, the protein was isolated and the sequence of a proteolytic fragment was determined. The peptide unambiguously matched an uncharacterized ORF in S. cerevisiae, hereafter referred to as *HAT2* (*H*istone *A*cetyl*t*ransferase Subunit 2).

By protein sequence comparison, *HAT2* was most

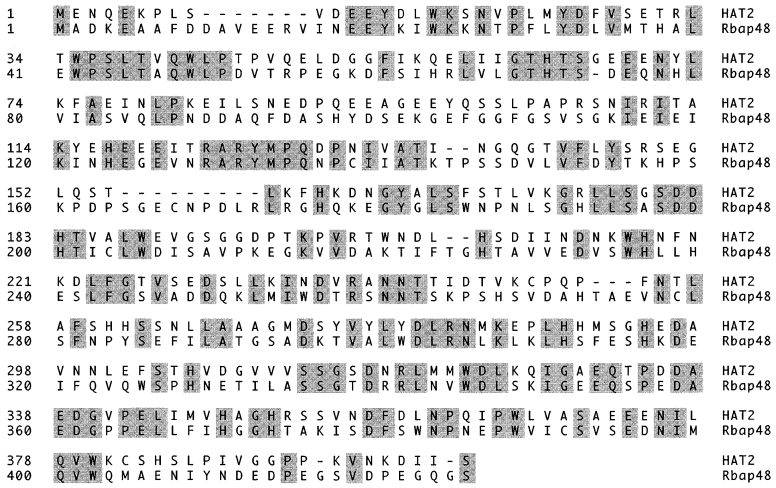

Figure 4. *HAT2* Is Highly Similar to Human Rbap48

A comparison of the amino acid sequences of *HAT2* and Rbap48. Identical amino acids are shaded. The WD repeats in HAT2 are located at the following positions: 57–88, 116–145, 159–189, 207–237, 251–280, 294–324, and 351–381.

similar to two nearly identical human proteins, Rbap48 and Rbap46, which were originally isolated based on their ability to bind the retinoblastoma protein (Rb) (BLAST score of 10^{-105} (Altschul et al., 1990; Qian et al., 1993; Qian and Lee, 1995). Rbap48, Rbap46, and Hat2p share seven WD repeat motifs (Neer and Smith, 1996), but the striking similarity between Hat2p and Rbap48 was also observed in regions of the proteins outside of these repeats (Figure 4).

Rbap48 is identical to the p48 subunit of the human chromatin assembly factor complex, CAF-1 (Verreault et al., 1996 [this issue of *Cell*]). Also, the Rbap48 protein copurifies with a recently isolated human histone deacetylase catalytic subunit, HD1 (Taunton et al., 1996). Thus, the Hat2p/Rbap48 family of proteins has now been found to copurify with three separate protein complexes that are involved in distinct aspects of histone metabolism.

Hat1p and Hat2p Are Physically Associated

While the Hat2p/Rbap48 family copurify with various histone-related activities, they have not been shown to be physically associated with these protein complexes. Therefore, we tested whether Hat1p and Hat2p are physically associated. The *HAT2* ORF was fused to a sequence encoding a peptide that contained both a hemagluttinin epitope (HA) and six histidines (His_6). This chimeric protein was expressed in yeast, which permitted visualization of Hat2p with α-HA antibodies and rapid isolation of Hat2p via affinity chromatography through the His_6 residues.

Cytoplasmic extracts were made from yeast strains expressing either the tagged Hat2p or a vector control. These extracts were then passed over His_6 affinity columns. As can be seen in Figure 5A, the HA-His_6-Hat2p fusion protein binds quite well to the affinity column.

The presence of Hat1p was monitored in the His_6 affinity column fractions both by Western blots probed with an affinity purified α-Hat1p antibody (middle panel, Figure 5A) and by HAT activity (bottom panel, Figure 5A). In the absence of HA-His_6-Hat2p, neither Hat1p nor HAT activity bound the HIS_6 affinity column. However, when HA-His_6-Hat2p is expressed, Hat1p and the HAT activity were found in the bound fraction. This result strongly suggests that Hat1p and Hat2p are physically associated. Whether Hat1p and Hat2p are in direct physical contact cannot be proven by this type of analysis, but the observation that only Hat1p and Hat2p were present after the final step of the cytoplasmic HAT purification argues for a direct interaction (Figure 2B).

If Hat1p and Hat2p are physically associated, then they should exist in a heteromeric complex. To test this prediction, the apparent molecular weight of Hat1p was ascertained by gel filtration chromatography as a function of *HAT2*. In a wild-type extract Hat1p had an apparent molecular weight >200 kDa (Figure 5B, top). However, when the *HAT2* gene was deleted, the apparent molecular weight of Hat1p was $\sim$45 kDa, consistent with it being in a monomeric form (Figure 5B, bottom). Together, these results indicate that Hat2p and Hat1p are physically associated in a large (>200 kDa) complex.

Hat2p Is Required for Full Hat1p Activity

From the data presented above, Hat1p and Hat2p exist in a protein complex. However, does the presence of Hat2p influence the activity of Hat1p? To answer this question, Hat1p from wild-type and *hat2* strains was partially purified and assayed for HAT activity. Using histones as a substrate, the specific activity of Hat1p isolated from a *hat2* strain was approximately 10-fold lower than from a wild-type strain (Figure 6A) while the specificity of the enzyme for histone H4 is unchanged (data not shown). A 10-fold decrease in specific activity was also observed when a synthetic peptide encoding the NH_2-terminal 28 amino acids of yeast histone H4 was used as the substrate for Hat1p (Figure 6B). Specifically, Hat1p from the *hat2* strain incorporated 10-fold less ^3H into the lysine at position 12 than Hat1p isolated from a wild-type strain. From these data it appears that the association of Hat1p with Hat2p is functionally significant and required for full activity of the Hat1p enzyme. However, the absence of Hat2p does not significantly affect the specificity of Hat1p as Lys-12 of histone H4 is still the only site modified by the enzyme.

A Hat1p/Hat2p Complex Is Required for Stable Histone H4 Tail Binding

There are two simple models to explain the reduced specific activity of Hat1p in the absence of Hat2p. Hat2p

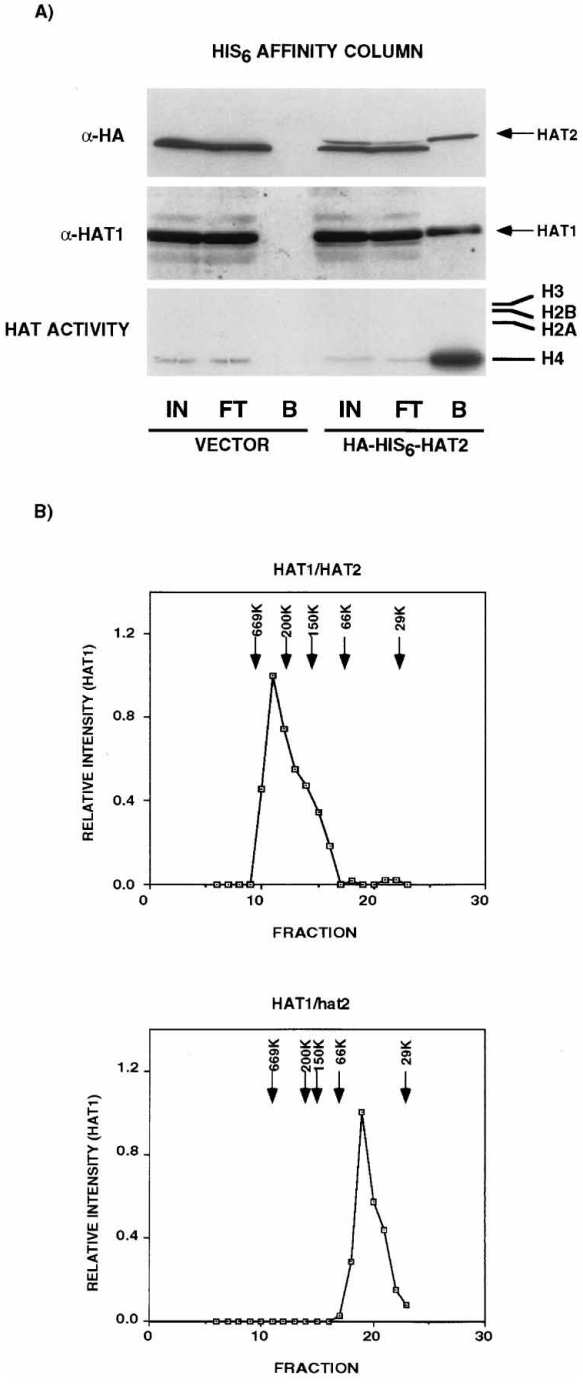

Figure 5. Hat1p and Hat2p Are Physically Associated

(A) Yeast cytoplasmic extracts prepared from strains containing either vector alone or a vector expressing an HA-His_6 tagged Hat2p were passed over a metal chelate column. Proteins bound to the resin were eluted with buffer containing 100 mM imidizole. Aliquots (50 μL) of the input (IN), flow through (FT), and bound (B) fractions were analyzed by probing a Western blot with α-HA and α-Hat1p antibodies as indicated. Hat2p and Hat1p are indicated by arrows. The faster migrating band visualized by the α-HA antibody is an unrelated yeast protein that cross-reacts with α-HA antibodies. The input, flow through, and bound fractions (10 μL) were also incubated with ^{3}H-acetyl CoA and chicken erythrocyte histones, and the reaction products were resolved by SDS–PAGE and fluorography (HAT ACTIVITY). The mobility of the core histones, as determined by Coomassie blue staining, is indicated. (B) Equivalent fractions of a DE-52 column isolated from a *HAT2* wild-type strain (top) and a *hat2* mutant strain (bottom) were chromatographed on a Superdex 200 gel filtration column. The presence of Hat1p was determined by quantitation of Western blots probed with affinity purified α-Hat1p antibodies (RELATIVE INTENSITY). The elution of molecular weight standards (in kDa) is indicated by the arrows.

A)

STRAIN	[Hat1p]	ACTIVITY	SPECIFIC ACTIVITY
YPH250 (HAT2)	1.00	31.7	31.7
UCC623 (hat2)	1.06	3.3	3.1

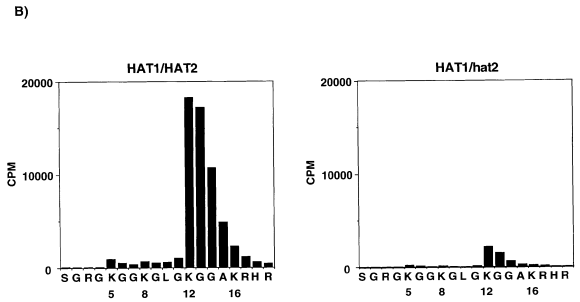

Figure 6. *HAT2* Is Required for Full Hat1p Activity

Yeast cytoplasmic extracts were prepared from strains YPH250 (*HAT2*) and UCC623 (hat2) and Hat1p was partially purified from each strain by DE-52 chromatography.

(A) The relative concentration of Hat1p in fractions from both strains ([Hat1p]) was determined by quantitation of a Western blot probed with α-Hat1p antibodies. The concentration of Hat1p in the YPH250 fraction was arbitrarily set at 1.00. The activity from an equivalent fraction from each strain was determined by measuring the incorporation of ^{3}H-acetyl groups into chicken erythrocyte histones under the HAT assay conditions (Experimental Procedures). The activity is expressed as femtomoles ^{3}H-acetyl groups incorporated per minute. The specific activity is given as femtomoles ^{3}H-acetyl groups incorporated/minute/[Hat1p].

(B) Equal amounts of Hat1p isolated from YPH250 (*HAT2*) and UCC623 (hat2) were incubated with ^{3}H-acetyl CoA and a synthetic peptide that contained the NH_2-terminal 28 amino acids of yeast histone H4. The peptide was analyzed as described in Figure 1.

may either augment the catalytic properties of Hat1p, or it may aid in the interaction of Hat1p with its substrate, histone H4. To test this latter idea, cytoplasmic extracts from wild-type, *hat1*, or *hat2* cells were mixed with the histone H4 tail affinity resin, and the ability of Hat1p and Hat2p to bind to histone H4 was determined. Under identical buffer conditions, only the Hat1p/Hat2p complex was capable of binding to this column (Figure 7). In the absence of Hat2p, Hat1p was not stably bound by the histone H4 tail. Similarly, Hat2p was not bound without Hat1p (Figure 7). These results support the idea that Hat2p facilitates the binding of Hat1p to the histone H4 tail, consequently increasing the specific activity of the HAT.

Negative Regulation of Hat1p Activity

While the binding to histone H4 and HAT activity of Hat1p in yeast cytoplasmic extracts was augmented by association with Hat2p, there were also indications that

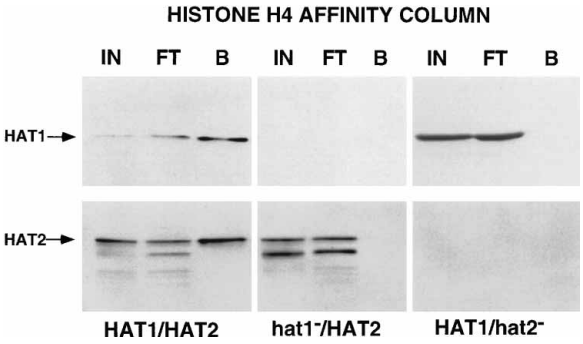

Figure 7. Hat1p and Hat2p Are Both Required for Histone H4 Binding

Cytoplasmic extracts, prepared from *HAT1*/HA-HIS$_6$-*HAT2*, *hat1*/HA-HIS$_6$-*HAT2*, and *HAT1*/*hat2* yeast strains were chromatographed on a histone H4 tail affinity column. Proteins able to bind the column were eluted with high salt (1 M NaCl). Aliquots of the input (IN, 2%), flowthrough (FT, 4%), and bound (B, 50%) fractions were analyzed on Western blots by probing with α-HA antibodies (top) and α-Hat1p antibodies (bottom). Hat1p and Hat2p are indicated by arrows.

Hat1p activity was negatively regulated in these extracts. Evidence for this came from examination of the substrate specificity of recombinant Hat1p (rHat1p) produced in E. coli. rHat1p retains the specificity for free histones that is seen in Hat1p isolated from yeast (data not shown). However, rHat1p acetylated histone H2A as well as histone H4 (Figure 8A). The rHat1p retains a strong preference for histone H4, but it has a distinct ability to acetylate histone H2A that is not seen with cytoplasmic extracts of yeast Hat1p.

This apparent "relaxed" specificity by Hat1p was not the result of an additional E. coli–derived activity, because extracts made from E. coli expressing a vector without the *HAT1* gene did not display HAT activity (data not shown). Conversely, yeast cytoplasmic extracts used in this study do not contain a deacetylase activity that removes the acetyl groups from histone H2A. When histones H4 and H2A, acetylated with rHat1p and ^{3}H-acetyl CoA, were incubated with yeast cytoplasmic extract, no decrease in the amount of either acetylated histone species was observed (data not shown). These results indicate that the rHat1p has a relaxed substrate specificity relative to crude yeast Hat1p and raises the possibility that the specificity of the enzyme isolated from yeast cytoplasmic extracts is a result of negative regulation.

Further evidence for negative regulation was obtained by determining the residues in the histone H4 tail that were acetylated by rHat1p. Contrary to the yeast derived enzyme, which only modified Lys-12, rHat1p acetylated both Lys-5 and Lys-12 (Figure 8B). Thus the Hat1p molecule has the intrinsic ability to acetylate histone H4 in the same diacetylation pattern that has been observed for cytoplasmic histone H4 in other eukaryotes (Chicoine et al., 1986; Sobel et al., 1995). However, it appears that the Lys-5 activity is inhibited in yeast cytoplasmic extracts.

Discussion

We have biochemically isolated the major cytoplasmic HAT activity of S. cerevisiae and identified its two main

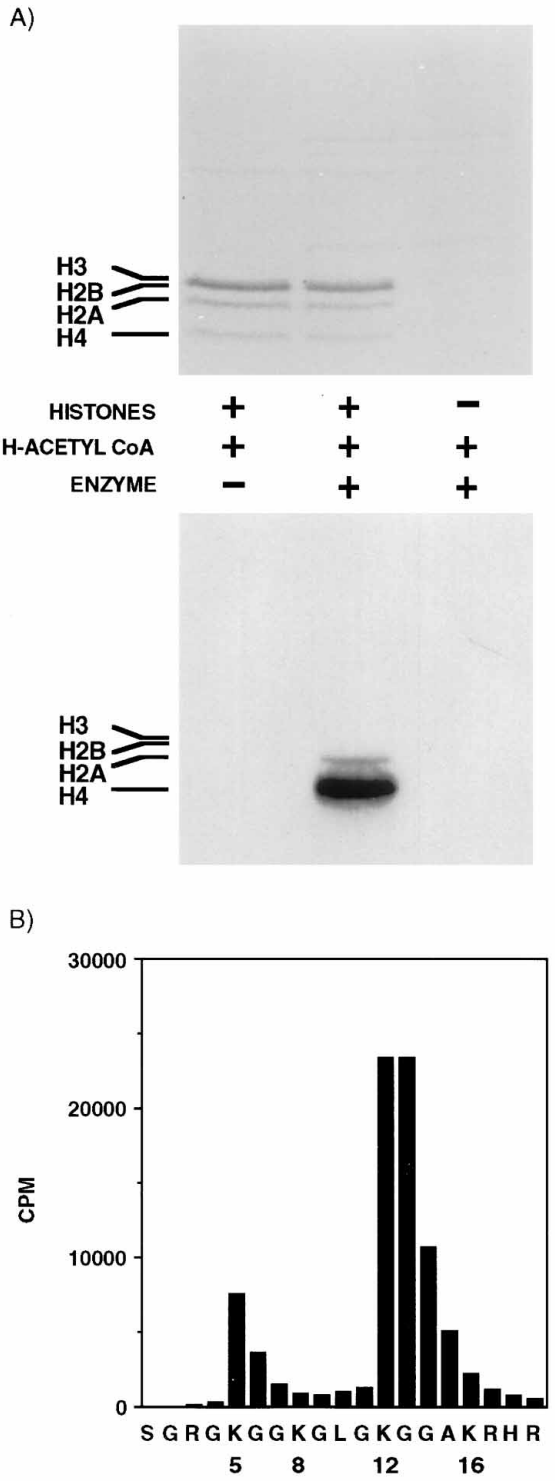

Figure 8. Recombinant Hat1p Has Altered Substrate Specificity

(A) rHat1p was incubated with ^{3}H-acetyl CoA and chicken erythrocyte histones as described in Figure 1. The reaction products were resolved by SDS–PAGE, the gel was stained with Coomassie blue (top panel) and then analyzed by fluorography (bottom panel). The mobility of each of the core histones is indicated along the left side of the panels.

(B) rHAT1p was incubated with ^{3}H-acetyl CoA and a synthetic peptide that contained the NH_2-terminal 28 amino acids of yeast histone H4. The peptide was analyzed as described in Figure 1.

components. *HAT1* encodes the catalytic subunit, and *HAT2* encodes a protein required for high affinity binding of the acetlytransferase to histone H4. These findings and the accompanying results provide new insights into the acetylation and general metabolism of histones.

Specificity of the Major Yeast B-type Histone Acetyltransferase

The identification of *HAT1* has allowed us to demonstrate that a single enzyme isolated from yeast cytoplasm specifically acetylates free histone H4 at Lys-12 (Figure 1). Yet interestingly, Hat1p has the intrinsic ability to acetylate histone H4 at Lys-5 and Lys-12 (Figure 8). These are the same sites where newly synthesized histone H4 is acetylated in the cytoplasm of HeLa, Drosophila, and Tetrahymena cells (Chicoine et al., 1986; Sobel et al., 1995). There are several implications of these findings. Cytoplasmic Hat1p may be modified such that its activity is limited to acetylation of Lys-12 of histone H4. Hat2p does not appear to be involved in this modification since in the absence of *HAT2*, Hat1p still only acetylates Lys-12 of H4 (Figure 6). Rather a more subtle change is likely to impart specificity (e.g. covalent modification, association with a small molecule, protein folding), because without Hat2p, Hat1p appears to be a simple monomer in the cytoplasm (Figure 5).

If histone H4 is acetylated at Lys-5 and Lys-12 in the yeast cytoplasm as it is in other organisms, then there may be a second form of cytoplasmic Hat1p that acetylates Lys-5 but was not detectable under our isolation/assay conditions. Alternatively, the *HAT1*-independent histone H4 acetyltransferase activity we detected may acetylate Lys-5 of H4 in the yeast cytoplasm (Figure 3).

The isolation of *HAT1* and the ability to express it in E. coli (rHat1p) have allowed us to deduce a primary recognition sequence for the enzyme's intrinsic activity. Only three proteins/peptides were acetylated by rHat1p: chicken/yeast histone H4, Tetrahymena H4, and chicken H2A (Figure 8 and data not shown). (Tetrahymena has several amino acid substitutions and the 3rd residue is deleted relative to most other histone H4 sequences.) While there are multiple lysines in these polypeptides, only residues 5 and 12 of yeast histone H4 and 11 of Tetrahymena H4 were acetylated. By comparing primary sequences in the vicinity of these acetylated lysines, a common motif was identified: GXGKXG (yeast/chicken H4 Lys-5: GRGKGG, Lys-12: GLGKGG; Tetrahymena H4 LYS-11: GMGKVG). While we have not directly determined the residue in chicken histone H2A that is modified by rHat1p, we predict that it is Lys-5. This residue falls within the predicted recognition motif (GRGKQG) and is the only site in chicken H2A that is found to be acetylated in vivo (van Holde, 1989). Furthermore, the recognition motif was not present at any of the nonacetylated lysines in proteins tested as substrates (e.g. Lys-8, 16, 20 in yeast H4; Lys-4, 9, 15 in Tetrahymena H4).

Histone Acetyltransferases in Yeast

In yeast, Hat1p exists in a multisubunit complex, in which the enzyme has its greatest activity. The only other catalytic HAT identified in yeast, Gcn5p, also exists in a heteromeric complex, with Ada2p and Ada3p (Georgakopoulos et al., 1995; Horiuchi et al., 1995; Brownell et al., 1996). While it is not known whether Ada2p and Ada3p are involved in the HAT activity of Gcn5p, it seems likely they too play a role in regulation or specifity, similar to the interplay between Hat1p and Hat2p. Thus, activity and specificity of HATs may be dependent on their association with other proteins. Given this idea, it is tempting to speculate that Hat1p also resides in the nucleus and acetylates histone H4 in chromatin, when complexed with proteins other than Hat2p that convert Hat1p to a type A HAT. In fact, Hat1p is detected in nuclear extracts by Western blotting (data not shown).

The Role of Hat2p in Histone Metabolism

Hat2p is an important component of the major cytoplasmic HAT complex in yeast. In addition, its similarity to the human protein Rbap48, which is a component of the chromatin assembly factor CAF-1 and the histone deacetylase HD1, implies that Hat2p-like proteins may participate in the same histone-related events in S. cerevisiae (Taunton et al., 1996; Verreault et al., 1996). For instance, we predict Hat2p/Rbap48 family proteins interact with Rpd3p, the yeast homolog of HD1, and thus also are involved in histone deacetylation in yeast (Vidal and Gaber, 1991; Taunton et al., 1996).

The interesting link shared by these Hat2p/Rbap48-associated activities is their interaction with histone H4. Both Hat1p and HD1 act on histone H4, and CAF-1 interacts with a complex of histones H3 and H4. We propose that Hat2p/Rbap48 family proteins escort the catalytic subunits of these various activities to histone H4. Hat2p is an escort in the sense that, alone, it is unable to bind to H4 (Figure 7), requiring its catalytic partner, Hat1p, for high affinity interaction with the histone tail.

Currently it seems that Hat2p/Rbap48 family proteins will be in independent partnerships with each of their associated activities. For instance, it is difficult to reconcile the antagonistic activities of Hat1p and Rpd3 (HD1) in the same macromolecular complex. However, Hat2p may provide an interesting link between the cytoplasmic acetylation of histone H4 and the subsequent fate of H4: deposition onto replicated DNA. Both acetylated histone H4 and a Hat2p/Rbap48 family protein are part of the chromatin assembly complex (Verreault et al., 1996). Following assembly into chromatin, the cytoplasmically acetylated histone H4 is rapidly deacetylated (Ruiz-Carrillo et al., 1975), a process that, given the association of Rbap48 with a histone deacetylase, could potentially involve Hat2p.

Curiously, there is no obvious phenotypic consequence of deleting *HAT1*, *HAT2*, or both genes from yeast cells. Such a lack of effect is likely explained by an apparent redundancy of HAT activity in yeast. For example, even in the absence of *HAT1* there are still HAT activities in the cytoplasm. *HAT2* has an apparent homolog in yeast, *MSI1*, which may substitute for its role in some processes. Another possible explanation for the lack of phenotypic consequence of *HAT1* or *HAT2* deletions is that acetylation of the histone H3 NH_2 terminus may compensate for loss of H4 acetylation. It has been shown that the presence of the NH_2-terminal tails

of either histone H3 or H4 is sufficient for cell viablity in yeast; only deletion of both is lethal (Morgan et al., 1991; Ling et al., 1996). In biochemical experiments, either the H3 or H4 terminus is sufficient for assembly of histones into nucleosomes; only the lack of both tails prevents assembly (Ling et al., 1996). Thus, there may be multiple pathways by which histones are prepared for assembly into nucleosomes on the newly replicated DNA fiber. The identification of *HAT1* and *HAT2* as components of the major cytoplasmic HAT activity of yeast provides an entry point into a mechanistic understanding of histone acetylation and nucleosome assembly.

Experimental Procedures

Yeast Strains and Cell Growth

Yeast strain PKY501 was used for purification of proteins (Kayne et al., 1988; Durrin et al., 1991). Cells were grown in 2 l cultures of 1% yeast extract, 2% glucose, and 50 μg/ml ampicillin to late log phase ($OD_{660} = 1$). They were harvested by centrifugation at 4000 × g for 5 min.

The *HAT1* gene was disrupted in the diploid strain UCC3542 (*MAT***a**/*MAT*α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 leu2-Δ1/leu2-Δ1 TRP1/trp1-Δ63 his3-Δ200/his3-Δ200 adh4::URA3/adh4::URA3* [*URA3* at telomere VII-L] *DIA5-1/DIA5-1* [*ADE2* at telomere V-R] *PPR1/Δppr1::LYS2). UCC622 was generated by transforming YPH250 (MAT***a** *ura3–52 lys2–801 ade2–101 trp1-Δ1 his3-Δ200 leu2-Δ1* (Sikorski and Hieter, 1989; Innis et al., 1990) with pΔHAT1::HIS3 cleaved with AflII and EcoRI and selecting for His^+ colonies. UCC623 was made by transforming YPH250 with pSD91 digested with NotI and PstI and selecting for Trp^+ colonies. UCC624 was made by transforming UCC622 with pSD91 digested with NotI and PstI and selecting for Trp^+ colonies. The desired transformants were confirmed with Southern blots. UCC625 and UCC626 were made by transforming UCC623 and UCC624, respectively, with pPK90.

Plasmids and DNA Constructs

The *HAT1* gene was cloned by polymerase chain reaction (PCR) using PKY501 genomic DNA as template and TAQ DNA polymerase (Innis et al., 1990). The PCR primer for the 5′ end of the gene was 5′ GGCATGCCATATGTCTGCCAATGATTTCAAG 3′, which incorporates an NdeI site upstream of the *HAT1* coding sequence. The primer for the 3′ end of the gene was 5′ GCGGAATTCCTTATTTCAG GCTTGTTAAAC 3′, which incorporated an EcoRI site downstream of the *HAT1* coding sequence. The *HAT1* PCR product was digested with these enzymes and then ligated into the corresponding sites of plasmid pT7SC (United States Biochemical) to create pT7Sc-HAT1. This plasmid was then transformed into E. coli strain BL21(DE3)pLYSS (Studier and Moffatt, 1986), and *HAT1* protein expression was induced with IPTG. The accuracy of the PCR-generated *HAT1* ORF was verified by DNA sequencing.

The *HAT1* gene was replaced with the *HIS3* gene either by PCR or plasmid-mediated transformation. For the PCR method, the primers 5′ CAAATAAATATGTTATTATATATTTAATAAACAGTTAGATCCT GCCTCGGTAATG 3′ and 5′ ATTATGCTTAAGCTATAACTATAGTGAG AATCAAGAATTCGTTCAGAATGACACG 3′ were used (Baudin et al., 1993). A plasmid used to disrupt the *HAT1* gene, pΔHAT1::HIS3, was created by replacing the 631 bp BglII/BamHI fragment in the middle of the open reading frame with the *HIS3* gene as a BamHI fragment from YDpH (Berben et al., 1991).

Epitope-tagged *HAT2* was expressed in yeast using plasmid pPK90 (generously provided by Dr. Paul Kaufman). pPK90 contains the *HAT2* gene on a 4.0 kb EcoRI–SpeI restriction fragment from yeast genomic clone #2340 (ATCC) cloned into EcoRI–XbaI digested pRS416 (Sikorski and Boeke, 1991). A BamHI restriction site was inserted immediately upstream of the *HAT2* stop codon by PCR. A cassette encoding a hemagluttinin epitope followed by six histidine residues (Gavin et al., 1995) was inserted into this BamHI site. DNA sequence analysis confirmed that the protein encoded will contain all Hat2p residues followed by GSYPYDVPDYAHHHHHHGS. The *HAT2* ORF plus some additional 5′ and 3′ noncoding sequence were amplified from yeast genomic DNA using the following primers: 5′ GGAATTCCATG-CTCCTGTAGGCTCAGGAG 3′ and 5′ CGGGAT CCT-TTTATTCAATGTGCTCAACC 3′. This PCR fragment was digested with EcoRI and BamHI and subcloned into pVZ1 (Henikoff and Eghtedarzadeh, 1987) cut with the same enzymes to create pSD90. The MscI–StyI fragment of the *HAT2* gene was replaced with a blunt-ended BamH I fragment from YDpW (Berben et al., 1991) containing the *TRP1* gene to create pSD91 (pSD90 and pSD91 were provided by Scott Diede).

Preparation of Cytoplasmic Extract

All purification procedures were performed at 4°C, and each buffer contained 1 mM PMSF, 5 mM benzamidine, and 0.1 mM DTT unless otherwise indicated. Spheroplasts were prepared immediately after harvesting cells as previously described (Lowary and Widom, 1989). Spheroplasts were lysed by resuspension in 50 ml lysis buffer (10 mM sodium cacodylate [pH 6.0], 18% (wt/vol) Ficoll 4000) followed by dilution with 100 ml buffer A (10 mM sodium cacodylate [pH 6.0], 50 mM NaCl, 1.0 mM $MgCl_2$). Nuclei and cell debris were pelleted by centrifugation at 1500 × g for 10 min.

Histone Acetyltransferase Purification

DE-52 Chromatography

Cytoplasmic extract was centrifuged at 10,000 x g for 10 min. The resulting clarified extract was applied to a DE-52 (Sigma) column (0.3 ml bed volume/g cells) equilibrated with DN(50) (DN buffers contain 25 mM Tris [pH 7.5], 0.1 mM EDTA, 10% glycerol, and the concentration of NaCl [in mM] is listed in parenthesis). The column was then washed with 5 column volumes of DN(50). Proteins were eluted with a 5 column volume gradient from DN(50) to DN(500) followed by a 3 column volume wash with DN(500).

Ammonium Sulfate Precipitation

Peak fractions of activity from the DE-52 column were pooled and 0.291 g $(NH_4)_2SO_4$/ml sample was added. The precipitate was pelleted by centrifugation at 10,000 × g for 10 min, the supernatant was recovered, and 0.145 g $(NH_4)_2SO_4$ was added per milliliter. Precipitated proteins were pelleted at 10,000 × g for 10 min. The pellet was resuspended in I(0) buffer (25 mM Imidazole–HCl [pH 7.4], 0.1 mM EDTA, 10% glycerol, and the concentration of NaCl [in mM] is listed in parenthesis) to a total volume of ~1 ml/100 g cells.

Gel Filtration Chromatography

Aliquots (200 μl) of the resuspended ammonium sulfate pellet were fractionated on a Superdex 200 column (Pharmacia) in I(50) buffer. The column was run on an FPLC (Pharmacia) with a flow rate of 0.25 ml/min and 0.5 ml fractions were collected. For analytical gel filtration chromatography, samples were mixed with an equal volume of gel filtration molecular weight standards (GFMW, Sigma) before loading on the column. The elution profiles of the molecular weight standards were determined by examination of Coomassie blue–stained protein gels.

Chromatofocusing Chromatography

Peak fractions of activity from the Superdex 200 column were pooled and applied directly to a PBE94 (Sigma) chromatofocusing column (1 ml bed volume/25 g cells) that had been equilibrated with I(0). Following a 5 column volume wash with I(0), a 20 column volume pH 7.0 to pH 4.0 gradient was developed on the column using polybuffer 74 (Sigma) diluted 1/8 with ddH_2O and adjusted to pH 4.0 with HCl. The column was then returned to pH 7.0 by washing with DN(50). Protein remaining on the column was then eluted in successive steps with DN(400) and DA(2000) (DA buffers are identical to DN buffers except that $(NH_4)_2SO_4$ is substituted for NaCl).

Phenyl Sepharose Chromatography

Peak fractions from the chromatofocusing column were pooled and applied directly to a phenyl sepharose (Sigma) column (1 ml bed volume/25 g cells) that had been equilibrated with DA(1000). Buffers used hereafter do not contain DTT. The column was washed with 10 column volumes of DA(1000). Proteins were then eluted with a 10 column volume gradient from DA(1000) to DA(0) followed by a 5 column volume wash with DA(0).

Histone H4 Affinity Chromatography

Histone H4 affinity resin was produced by attaching a 29 amino acid synthetic peptide encoding the N-terminus of yeast histone H4 that

included a cysteine residue at the C-terminus (generously provided by Dr. Roger Kornberg) to activated thiol Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Acetylated histone H4 affinity resin was produced by mixing the histone H4 affinity resin with an equal volume of saturated sodium acetate and then adding an amount of acetic anhydride equal to the weight amount of peptide and incubating the mixture for 1 hr at 4°C (Means and Feeney, 1971). The resin was then washed extensively with DN(50). A 0.5 ml acetylated histone H4 N-terminal peptide affinity column was followed in series by a 0.5 ml unmodified histone H4 peptide column and both were equilibrated with 10–20 ml of DN(50). Peak fractions of activity from the chromatofocusing column were pooled and applied directly to the columns. The histone acetyltransferase activity flows through the acetylated histone H4 tail affinity resin and is bound to the unmodified column. The columns were washed with 10 ml of DN(50), then separated, and the unmodified histone H4 column was washed with another 10 ml of DN(50). The column was eluted with a 5 ml gradient from DN(50) to DN(1000) followed by a 5 ml wash with DN(1000).

Metal Chelate Affinity Chromatography

Talon (Clonetech) affinity resin was equilibrated with TN(50) buffer (TN buffers are identical to DN buffers but do not contain EDTA). Cytoplasmic extracts were incubated in batch with Talon affinity resin with constant mixing for 30 min (5 ml extract/0.5 ml resin). The resin was pelleted by centrifugation at 700 × g for 2 min and the liquid was decanted. The resin was then washed twice with 10 column bed volumes of TN(50). The resin was loaded into a column and washed with another 10 column bed volumes of TN(50). Bound proteins were then eluted with TN(50) buffer containing 100 mM imidizole.

Protein Sequencing

Peak fractions from the histone H4 affinity column were pooled and concentrated in a Centricon 10 (Amicon). The sample was then electrophoresed on an SDS-polyacrylamide gel, and proteins were visualized by staining with Coomassie blue and then briefly destaining with 50% methanol (~5 minutes). The 42 and 50 kDa protein bands were excised with a razor. Each protein gel slice was then incubated with trypsin and the resulting peptides were isolated for sequencing as described ([Williams and Stone, 1995], Keck Biotechnology Center, Yale University). Two peptide sequences were obtained from the 42 kDa protein (peptide 1: YDALDQRDPEK, peptide 2: EYSLNGEEFVVYK), both of which correspond to the same S. cerevisiae ORF (accession number S52530). The peptide sequence: EILSNEDPQEEAGEEYQSSLPAP was obtained from the 50 kDa protein. This sequence uniquely identifies an ORF (accession number P38079) in the S. cerevisiae genome.

Synthetic histone H4 peptides incubated with ^{3}H-acetyl Coenzyme A and HAT activity were separated from the majority of protein contaminants after the reaction by passage through a Microcon 10 (Amicon). The peptides were then subjected to N-terminal peptide sequencing with 50% of each cycle used for amino acid identification and 50% analyzed by scintillation counting (Sobel et al., 1994).

Production of rHat1p

Cultures E. coli strain BL21(DE3)pLYSS containing either pT7SC or pT7SC-HAT1 were grown at 37°C to an OD_{600} of 0.4–0.5. IPTG was added to a final concentration of 0.4 mM, and the cultures were allowed to grow for an additional 3 hr. The cells were harvested by centrifugation at 5000 × g for 5 min. Cells were resuspended in 0.1 volumes of DN(50) plus 0.1% NP-40. The cells were lysed by three freeze-thaw cycles using liquid N_2. The cell lysate was sonicated briefly to reduce the viscosity of the solution and then centrifuged at 10,000 × g for 10 min to pellet insoluble material. The supernatant contained the bulk of the rHat1p and was used for the HAT assays.

Histone Acetyltransferase Assays

Liquid histone acetyltransferase assays were performed in a final volume of 50 μl in buffer DN(150). Reactions also contained 0.1 μM ^{3}H acetyl Coenzyme A (6.1 Ci/mmol, ICN) and ~1 mg/ml chicken erythrocyte core histones. Chicken erythrocyte core histones were purified as previously described (Feng et al., 1993). Chromatin was isolated as described (Widom, 1986). Reactions were incubated at 37°C for 30 min, and then transferred to P-81 filters (Whatman). The filters were washed three times with 250 ml of 50 mM $NaHCO_3$ (pH 9.0) for 10 min. They were then rinsed with acetone and air dried. The amount of ^{3}H bound to the filters was quantified by liquid scintillation counting. Histone acetyltransferase activity gel assays were performed as described (Brownell and Allis, 1995).

Protein Gels

Enzyme samples were run on 5% stacking, 10% resolving SDS-polyacrylamide gels (30:0.8 acrylamide:bis-acrylamide) (Laemmli, 1970). Histones were electrophoresed on 7% stacking, 18% resolving gels (30:0.8 acrylamide:bis-acrylamide). Gels containing ^{3}H-labeled histones were incubated in Fluorohance (RPI) prior to drying and autoradiography.

For Western blots, protein gels were transferred to nitrocellulose using a semi-dry transfer apparatus (Biorad [Harlow and Lane, 1988]). The blots were blocked and probed using standard procedures (Harlow and Lane, 1988). Western blots were visualized using an ECL chemiluminescent detection kit according to the manufacturer's instructions (Amersham). Western blots were quantified following scanning of the autoradiographic film using the NIH image 1.59 program.

Acknowledgments

We would like to thank R. Kornberg for the gift of yeast histone H4 peptide, C. D. Allis for providing Tetrahymena histone peptides and for communicating the HAT activity gel assay protocol prior to publication, M. Grunstein for strain PKY501, S. Diede for plasmids pSD90 and pSD91, P. Kaufman for plasmid pPK90, P. Lowary for chromatosomes and core particles, and B. Stillman for discussions and communicating the sequence of CAF-1 p50 prior to publication. We would also like to thank S. Diede, M. Singer, A. Kahana, and B. Berg for critical reading of the manuscript. This work was supported by postdoctoral fellowships from the American Cancer Society and the National Institutes of Health to M. R. P., a PEW Charitable Trust Biomedical Scholars Fellowship, a Fletcher Fellowship from the Cancer Research Foundation, grant GM43893 from the National Institutes of Health to D. E. G., and grant GM50932 from the National Institutes of Health to J. W. This work was done while M. R. P. and D. E. G. were at the University of Chicago.

Received June 19, 1996; revised August 9, 1996.

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